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(54) Overproduction of riboflavin in yeast

(57) The present invention is directed to a yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promotor functional in such yeast strain, a process for the production of riboflavin characterized therein that such a yeast strain is cultured under suitable culture conditions and process for the production of a food or feed composition

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Description

[0001] Derivatives of riboflavin (the flavocoenzymes FMN and FAD) are universally required for redox reactions in all cellular organisms. Riboflavin (vitamin B_2) is produced by all plants and by many microorganisms [Demain A.L. Riboflavin oversynthesis. Ann. Rev. Microbiol. 1972, 26, 369]. The compound is not produced in vertebrates. Riboflavin is therefore an essential nutrient for man and animals.

[0002] Riboflavin can be produced by chemical synthesis and by various fermentation procedures using strains of *Bacillus* (e.g. *Bacillus subtilis*), the ascomycetes *Ashbya gossypii* and *Eremothecium ashbyi* [Demain A.L. Riboflavin oversynthesis. Ann. Rev. Microbiol. 1972, 26, 369 and Mitsuda H, Nakajima K., Effects of 8-azaguanine on riboflavin production and on the nucleotide pools in non-growing cells of Eremothecium ashbyii. J. Nutr Sci Vitaminol (Tokyo) 1973; 19(3):215-227], various yeast strains such as *Candida guilliermondii*, *Candida famata* [F.W. Tanner, Jr.C. Vojnovich, J.M. Van Lanen. Riboflavin production by Candida species. Nature, 1945, 101 (2616):180-181]and related strains, as well as other microorganisms.

[0003] The pathway of riboflavin biosynthesis in yeast is shown in Fig. 1. The precursors for the biosynthesis of the vitamin are guanosine triphosphate (GTP) and ribulose 5-phosphate. One mol of GTP and two mol of ribulose 5-phosphate are required to biosynthetically generate one mol of riboflavin.

[0004] In the yeast, *Saccharomyces cerevisiae*, the biosynthesis of the vitamin requires at least six genes, specifically the genes *RIB1, RIB2, RIB3, RIB4, RIB5* and *RIB7* [Oltmanns O., Bacher A., Lingens F. and Zimmermann F.K. Biochemical and genetic classification of riboflavine deficient mutants of Saccharomyces cerevisiae. Mol. Gen. Genet. 1969, 105, 3061. In *C. guilliermondii*, the biosynthesis of riboflavin has also been shown to require the products of at least six genes, specifically the genes *RIB1, RIB2, RIB3, RIB4, RIB5* and *RIB6* (2). The enzymes specified by these *C. guilliermondii* genes and their roles in the biosynthetic pathway are summarized in Fig. 1. In contrast to the situation in *B. subtilis*, the riboflavin biosynthetic genes are not clustered in the eukaryots *S. cerevisiae* and *C. guilliermondii*.

[0005] The initial step in the biosynthetic pathway is the opening of the imidazole ring of GTP catalyzed by the enzyme, GTP cyclohydrolase II. The product of this enzyme has been reported to be 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate. This intermediate is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of side chain reduction, ring deamination, and dephosphorylation. The hypothetical enzyme involved in the dephosphorylation of 5-amino-6-ribitylamino 5'-phosphate is still unknown. The conversion of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to 6,7-dimethyl-8-ribityllumazine by the enzyme, 6,7-dimethyl-8-ribityllumazine synthase, requires a second substrate, 3,4-dihydroxy-2-butanone 4-phosphate, which is obtained from ribulose 5-phosphate by the catalytic action of 3,4-dihydroxy-2-butanone-4-phosphate synthase. Finally, 6,7-dimethyl-8-ribityllumazine is converted to riboflavin by a dismutation reaction catalyzed by riboflavin synthase. The sequence of the *RIB1* gene directing the synthesis of GTP cyclohydrolase II, the initial enzyme of the riboflavin pathway, has been established in the yeast, *C. guilliermondii* (4).

[0006] Recombinant strains of *Bacillus subtilis* for the production of riboflavin by fermentation have been described, e.g. in EP 405 370. These strains carry the riboflavin operon under the control of a strong promoter directing the production of the cognate enzymes in large amount. The gene constructs of the riboflavin operon under the control of a strong promoter can be present at one or several different locations on the *B. subtilis* chromosome. The incorporation of an additional gene of the riboflavin pathway under the control of a strong promoter at a separate locus on the *B. subtilis* chromosome has also been shown to increase the yield of riboflavin obtained by fermentation, see EP 821 063. bacteria

[0007] Whereas the production of riboflavin by strains of yeasts such as *C. guilliermondii* has been reported, recombinant DNA technology has not been applied for the overexpression of riboflavin biosynthetic genes in *C. guilliermondii* or in related flavinogenic yeasts so far.

[0008] It is therefore an object of the present invention to provide recombinant means which should allow the production of yeast strains which overproduce riboflavin. More specifically it is an object of the present invention to provide a yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon 1 expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promotor functional in such yeast strain and even more specifically such a yeast strain which belongs to the groups of flavinogenic yeasts which overproduce riboflavin under conditions of iron starva- 2 tion like a yeast strain which is selected from the following group: 3 Schwanniomyces, preferably Schwanniomyces occidentalis, Debaryomyces, preferably Debaryomyces kloeckeri, Torulopsis, preferably Torulopsis candida, or, especially Candida, preferably Candida guilliermondii or Candida famata (Logvinenko et al., Ukrainskii Biokhimicheskii Zhurnal 61(1), 28-32, 1989; Logvinenko et al., Mikrobiologiya 57(2), 181-186, 1988 and Nakase and Suzuki, Journal of General and Appl.Mikrobiology 31(1), 49-70 (1985). It is furthermore an object of the present invention into provide such yeast 5 strains wherein the polypeptide encoding DNA sequence is from yeast, preferably flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation, more preferably Candida, e.g. Candida guilliermondii or Candida famata. yeast DNA into yeast

[0009] It is also an object of the present invention to provide such yeast strains wherein th polypeptide ncoding DNA

sequence encodes a protein with GTP cyclohydrolase II activity and is selected from the following DNA sequences:

a) the DNA sequence as shown in Fig. 5 or its complementary strand;

b) DNA sequences which hybridize under standard conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and

c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

(6)

[0010] It is furthermore an object of the present invention to provide such yeast strains wherein the promotor is the TEF S. cerevisiae promotòr. (7)

[0011] It is also an object of the present invention to provide a process for the production of riboflavin characterized (8) therein that a yeast strain as described above is cultured under suitable culture conditions and the riboflavin produced is isolated from the medium or the yeast strain by methods known to the man skilled in the art, and a process for the (9) production of a food or feed composition characterized therein that riboflavin as obtained by such process is mixed with one or more suitable food or feed ingredients by a process known to the man skilled in the art.

[0012] All *C. guilliermondii* strains used in the practice of the present invention are derivatives of the *C. guilliermondii* strain obtained from the American Type Culture Collection (ATCC) under accession No. ATCC 9058 (1). Candida guilliermondii (ATCC 9058) has been redeposited as a Budapest Treaty deposit on April 1, 1998 and has been assigned accession No. ATCC 74437 *Candida guilliermondii* is a representative of yeast species which overproduce riboflavin (vitamin B2) under conditions of iron starvation.The group includes also *Schwanniomyces occidentalis*, (or called Debaryomyces occidentalis) *Debaryomyces cloeckeri*, *Torulopsis candida* and *Candida famata*. The latter species is used for industrial production of riboflavin. Regarding the taxomonic assignments of yeast species a man skilled in the art knows that these assignments are handled variably by different authors, for example: *Candida famatà - Debaryomyces hansenii - Torulaspora hansenii*, *Candida guilliermondii - Pichia guilliermondii - Yamadazyma guilliermondii* are used as synonyms. A man skilled in the art knows that microorganisms which can be used for the practice of the present invention, either as host cells or source for the isolation of DNA sequences, are available from depository authorities, e.g. the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) or the Deutsche Sammlung fur Mikroorganismen und Zellkulturen GmbH (DSM) or any other depository authority as listed in the Journal "Industrial Property" [(1991) 1, pages 29-40]. Pg 9058 Cf.

[0013] DNA sequences useful for the practice of the present invention and encoding a polypeptide with riboflavin biosynthetic activity can be obtained from any microorganism known to produce riboflavin (see above) in form of e.g., genomic or c-DNA sequences by methods known to the man skilled in the art or by using the wellknown PCR-Technology. The principles of the polymerase chain reaction (PCR) methode are outlined e.g. by White et al., Trends in Genetics, 5, 185-189 (1989), whereas improved methods are described, e.g. in Innis et al. [PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990)].

[0014] The sequence information needed for the design of the PCR-primers can be obtained from any sequence data base, for example like Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinston Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin, USA).

[0015] Once such DNA sequences have been obtained they can be expressed in any desirable host and the riboflavin biosynthetic activity of the encoded polypeptide can be determined by any assay known to the man skilled in the art and described, e.g. in Bacher A., G. Richter, H. Ritz, S. Eberhardt, M.Fisher and C. Krieger, Biosynthesis of riboflavin: GTP cyclohydrolase II, deaminase, and reductase. Methods in enzymology 1997; 280: 382-389; K.Kis, R. Volk and A. Bacher, Biosynthesis of riboflavin. Studies on the reaction mechanism of 6,7-dimethyl-8-ribityllumazine synthase. Biochemistry 1995, 34, 2883-2892; Logvinenko EM, Shavlovskii GM, Zakal'skii AE, Kontorovskaia Niu. Properties of 2,5-diamino-4-oxy-6-ribosylaminopyrimidine-5'-phosphate reductase, a enzyme of the second stage of flavinogenesis in Pichia guilliermondii yeast Ukr Biokkhim Zh 1989 Jul; 61(4): 47-54; G. Richter, M. Fischer, C.Krieger, S.Eberhardt, H. Lüttgen, I. Gerstenschläger and A. Bacher. Biosynthesis of riboflavin. Characterization of the bifunctional deaminase/reducase of *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 1997, 179, 2022-2028; K. Ritsert, D. Turk, R. Huber, R. Ladenstein, K. Schmidt-Bäse and A. Bacher. Studies on the lumazine synthase/riboflavin synthase complex of *Bacillus subtilis*. Crystal structure analysis of reconstituted icosahedral β subunit capsied at 2.4 Å resolution. J. Mol. Biol. 1995, **253**, 151-167.

[0016] The DNA sequences used for the practice of the present invention comprise at least one DNA sequence which enodes a polypeptide with riboflavin biosynthetic activity. It is however, understood by the man skilled in the art that also more then one, e.g. all enzymes of the riboflavin bipsynthetic pathway can be encoded by such DNA sequences and one or more of this enzymes can be ncoded by DNA sequences of different species origin or can be of partial or total

synthetic origin as long as they show at least one desired riboflavin biosynthetic activity. One example of such a DNA sequence is given in Fig. 5 coding for a GTP cyclohydrolase II. However, DNA sequences which hybridize under standard conditions to this DNA sequence and encode such a GTP cyclohydrolase are also useful for the practice of the present invention.

[0017] "Standard conditions" for hybridization in this context are conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, e.g. by Sambrook et al., "Molecular Cloning" second edition, Cold Spring Harbor Laboratory Press 1989, New York, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, e.g. in Sambrook et al. (s.a.). "Fragment of the DNA sequences" means in this context a fragment which codes for a polypeptide still having the enzymatic activity as specified above.

[0018] For the overexpression of the proteins encoded by the DNA sequences of the present invention these sequences can be linked to promoters which are functional in the desired yeast and are, e.g. the S.cerevisiae TEF-promotor (see Example 2) or the pho5-promotor [Vogel et al., Mol. Cell. Biol., 2050-2057 (1989); Rudolf and Hinnen, Proc, Natl. Acad. Sci. 84, 1340-1344 (1987)] or the gap-promotor or the aox1-promotor [Koutz et al., Yeast 5, 167-177 (1989; Sreekrishna et al., J. Basic Microbiol. 28, 265-278 (1988)] or the FMD promoter [Hollenberg et al., EP 299108] or MOS-promotor [Ledeboer et al., Nucleic Acids Res. 13, 3063-3082 (1985)].

TEF promoter
pho5 promoter
gap promoter
aox-1 promoter
fmd promoter
ARS elements

[0019] The DNA sequences useful for the practice of the present invention can also comprise so called "ARS" elements (autonomounsly replicating sequence) as described, e.g. in Example 1.

Examples

[0020] If not specifically indicated or referred to by references standard procedures have been used as described, e.g. in Sambrook et al. "Molecular Cloning", (s.a.) and Cregg, J.M., K.J. Barriner, A.Y.Hessler, and K.R. Madden (1985). Pichia pastoris as a host system for transformations. Mol. Cell. Biol. 5, 3376-3385.

Example 1

Autonomous replication of plasmid p19R1 in *C. guilliermondii*

rib1 → p19R1

[0021] The plasmid pFR1 carrying the RIB1 gene of *C. guilliermondii* has been described (Zakalsky at al. *Genetika* 26, 614-620, 1990).In order to subclone the RIB1 gene, plasmid pFR1 was digested with the restriction nuclease *Sal*I. The resulting fragments were cloned into the *Sal*I site of the pUC19 vector. The ligation mixture was transformed into the *E. coli* mutant strain BSV821 carrying a mutation of the *ribA* gene conducing to riboflavin deficiency. Colonies growing in the absence of riboflavin were isolated and were shown to contain a plasmid p19R1.

[0022] The plasmid p19R1 was sequenced and was shown to contain a 2.18 kb fragment of *C. guilliermondii* DNA in the *Sal*I site of the pUC19 vector. The sequence of this insert is shown in Fig. 2.

[0023] The DNA sequence shown in Fig. 2 carries the *RIB1* gene of *C. guilliermondii*. The plasmid transforms *C. guilliermondii* mutant defective in the *RIB1* gene to riboflavin prototrophy and can replicate autonomously in this yeast species. The replication was shown to be due to the presence of an autonomously replicating sequence (ARS) comprising approximately base pairs 1542 to 1755 in Fig. 2 and extending into the structural gene *RIB1*.

Example 2

Construction of a plasmid for hyperexpression of the *RIB1* gene of *C. guilliermondii*

[0024] The *TEF* gene of the yeast, *S. cerevisiae*, specifies the translation elongation factor 1-alpha. This gene is known to be transcribed in *S. cerevisiae* with high efficiency.

[0025] A DNA fragment carrying the *S. cerevisiae TEF* promoter and the 5' part of the the *RIB1* gene of *C.guilliermondii* was obtained by PCR amplification. Initially, a DNA sequence located upstream from the 5' end of the *S. cerevisiae TEF* gene was amplified by PCR with primers ShBle_V and TEF1_H using chromosomal DNA of *S. cerevisiae* as a template. The amplified DNA fragment (subsequently designeted *TEF* promoter) comprises bp 15 984 to 16 344 of the sequence listed under EMBL accession number gb/U51033/YSCP9513.

[0026] Independently, a DNA fragment comprising the 5'-terminal part of *C. guilliermondii* GTP cyclohydrolase II structural gene was obtained by PCR with primers PGgtpCY_V and PGgtpCY_nco using p19R1 plasmid as a template. The amplified DNA fragment (subsequently designeted 5'GTPcII) comprises bp 460 to 1145 of the sequence shown in Fig. 2.

[0027] The DNA amplificates obtained in th two PCR reactions described abov comprising parts of the *TEF* gene

of *S. cerevisiae* and of the 5'GTPcII were mixed, and a third PCR amplification was performed using the primers ShBle_V and PGgtpCY_nco. This reaction yielded a DNA fragment which contains the complete *TEF* promoter and the 5' part of the *C.guilliermondii RIB1* gene.

[0028] All primers used in these PCRs are shown in Table 2. The sequence of the final amplificate is shown in Fig. 3.

[0029] The amplificate contains a cutting site for the restriction nucleases *SphI* which had been introduced via the primer ShBle_V. The amplificate also contains an MscII site which is a feature of the RIB1 gene. The amplificate was digested with *SphI* and *MscI*.

rib1 (GTPase)

[0030] The plasmid p19R1 (whose constructions is described above) was also digested with the same enzymes, and the PCR amplificate was ligated into the digested plasmid.

p19R1

[0031] The ligation mixture was transformed into a mutant designated Rib7 of *Escherichia coli* carrying a mutation of the *ribA* gene which specifies GTP cyclohydrolase II. Transformation was performed by electroporation according to the protocol of Invitrogene (5).

[0032] The *E. coli* cells were plated on Luria-Broth plates supplemented with ampicillin (100 mg/ml) which did not contain riboflavin. Colonies growing on this medium were isolated and were shown to contain a plasmid designated pTC2.

[0033] The sequence of the insert of the plasmid pTC2 is shown in Fig. 3.

[0034] Plasmid pTC2 was digested with *XhoI* and *SalI* restriction endonucleases yielding 4.4 kb and 0.5 kb fragments. The 4.4 kb fragment was circularized with T4 DNA ligase. The ligation mixture was transformed into the Rib7 mutant of *E. coli* carrying the RibA mutation. Transformation was achieved by electroporation. The cells were plated on Luria-Broth plates containing ampicillin and no riboflavin (see above). Colonies growing on these plates were isolated and were shown to contain a plasmid pTCdXS2. The procedure had resulted in the removal of 0.5 kb base pairs from the plasmid pTC2.

[0035] The sequence of the insert of the plasmid pTCdXS2 is shown in Fig. 4. The open reading frame of the *RIB1* gene of *C.guilliermondii* is indicated. The DNA segment representing the promoter of the *TEF* gene of *S. cerevisiae* is also shown.

rib1 p.g.

Example 3

Construction of a recombinant *C. guilliermondii* strains

[0036] The riboflavin deficient mutant rh-21 with an apparent defect of the *RIB1* gene specifying GTP cyclohydrolase II (2) has been obtained after chemical mutagenesis of the L2 strain (2) which was previously obtained from the ATCC 9058 *C. guilliermondii* strain.

rh-21 p.g mutants

[0037] The plasmid pTCdXS2 was transformed into the *RIB1* mutant strain rh-21 of *C. guilliermondii* by the LiCl procedure, respectively (6). The cells were plated on YPD medium without added riboflavin. Colonies growing without riboflavin were isolated. They were monitored for GTP cyclohydrolase II activity and for riboflavin production as described below.

[0038] The prototrophic strains were monitored for the presence of DNA segments introduced with the plasmid by PCR analysis. PCR was performed using the primers ShBle_V and PGgtpCY_nco and boiled *C. guilliermondii* recombinant strains cells as template. Primer ShBle_V is complementary to the *TEF* promoter and primer PGgtpCY_nco is complementary to the *RIB1* structural gene. Amplificates of the expected length (1175 base pairs) were obtained from all transformants isolated. The amplificate obtained from strain XS-3 was isolated and was sequenced by the fluorescent dideoxy terminator method. The sequence is shown in Fig. 5. This sequence is identical with base pairs 1 to 1168 of the insert of plasmid pTCdXS2.

[0039] The recombinant transformants were genetically stable. Specifically, they did not segregate riboflavin deficient subclones.

Example 4

GTP cyclohydrolase activity in recombinant *C. guilliermondii* strains

[0040] The level of GTP cyclohydrolase II activity in the recombinant strains described above was determined as follows.

[0041] The recombinant *C. guilliermondii* cells were grown aerobically in synthetic Burkholder medium supplemented with trace elements (Science 101, 180, 1945) but without asparagine, during 2-3 days at 30°C. *C. guilliermondii* L2 strain (wild type) served as a control in these experiments.

[0042] Cells from exponential growth phase were harvested by centrifugation (5000 g, 15 min), washed twice with 20 mM Tris HCl, pH 8.2, containing 1 mM DTT and 1 mM $MgCl_2$. Cells were stored at -20°C. Frozen cells mass (1-3 g) was thawed in 3-9 ml of washing buffer. Cells were disrupted by agitation with glass beads (d = 0.8 mm). After centrifugation,

cell extract was dialyzed overnight against 100 volumes of washing buffer. Protein concentration was measured by the Lowry method.

[0043] Reaction mixtures for GTP cyclohydrolase assays contained 20 mM Tris HCl, pH 8.2, 3 mM DTT, 2 mM $MgCl_2$, 1 mM GTP, and protein (protein concentration, 1-3 mg/ml, total volume, 4 ml). They were incubated at 37°C for 20 min in the dark.

[0044] After incubation, 2 ml aliquots were removed, and 2,3-butanedione was added to a final concentration of 0.5 mg/ml. The mixtures were incubated at 95°C for 30 min. Blank values were processed in the same way but without added diacetyl.

[0045] Differences in specific fluorescence of both types of aliquots were determined and were used to calculate the concentrations of 6,7-dimethylpteridin and the activity of GTP cyclohydrolase II. Results are shown in Table 3.

[0046] Strain L2 from which the mutant rh-21 had been derived was used as a control. The enzyme activity in strain L2 was 2,9 nmol mg^{-1} h^{-1}. No enzyme activity was found in the riboflavin deficient recipient strain rh-21 carrying a mutation of the *RIB1* gene. The recombinant strains obtained by transformation with plasmid pTCdXS2 showed enzyme levels between 6,5 and 13,4 nmol mg^{-1} h^{-1} Thus, the enzyme level in recombinant strains was 2.3 - 4.6-fold higher as compared with the *C. guilliermondii* strain L2.

rib1 → pg

[0047] The activity of riboflavin synthase was also measured in the recombinant strains. The activity of riboflavin synthase was not affected by the transformation with the plasmids p19RI, pTC2, pTCdXS2. All strains analyzed had riboflavin synthase activities in the range of 20 nmol mg^{-1} h^{-1} (Table 3).

rib7 pg +

Example 5

Production of riboflavin by recombinant *C. guilliermondii* strains

pichia

[0048] *C. guilliermondii* strains (wild type and recombinant strains) were grown aerobically in synthetic Burkholder medium supplemented with trace elements [Science 101, p. 180, (1945)] but without asparagine during 4 days at 30 °C [F.W. Tanner, Jr.C. Vojnovich, J.M. Van Lanen, Riboflavin production by Candida species. Nature, 1945, 101 (2616): 180-181]. The suspension was centrifuged. Riboflavin concentration was determined fluorometrically. Results are shown in Table 4.

[0049] The wild strain L2 produced 1.2 mg riboflavin per liter under the conditions described. The recombinant strain XS-3 produced a 3-fold increased level of riboflavin (3.6 mg/l).

Example 6

Isolation of riboflavin

pichia

[0050] Two Erlenmeyer flasks (2.5 l) each containing 0.5 l of synthetic Burkholder medium containing trace elements but no asparagine were inoculated with the recombinant *C. guilliermondii* strain XS-3. The cultures were incubated with shaking at 30°C for 50 h. The solution was centrifuged. The supernatant was passed through a column of Florisil (4 ml bed volume) at a velocity of 500 ml/h. The column was washed with distilled water (7 ml). Riboflavin was eluted by a mixture of acetone/1M aqueous NH_4OH. The effluent was evaporated to dryness. The yield of riboflavin was determined photometrically.

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[0051]

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Table 1

Enzymes and genes of the riboflavin pathway				
	Enzyme	Gene		
		S. cerevisiae	*C. guilliermondii*	*E. coli*
A	GTP cyclohydrolase	*RIB1*	*RIB1*	*ribA*
B	bacterial deaminase			*ribD*
C	yeast reductase	*RIB7*	*RIB2*	
D	yeast deaminase	*RIB2*	*RIB3*	
E	bacterial reductase			*ribD*
F	unknown phosphatase			
G	lumazine synthase	*RIB4*	*RIB5*	*ribE*
H	riboflavin synthase	*RIB5*	*RIB7*	*ribC*
I	3,4-dihydroxy-2-butanon 4-phosphate synthase	*RIB3*	*RIB6*	*ribB*

Table 2

Nucleotide sequences of the primers used.		
N	**Primer**	**Sequence (5' - 3')**
1	ShBle_V	GGGCATGCAATTCGAGCTCGGTACCCG
2	TEB1_H	CGACTCACTATAGGAGGAAGCTTGGCGC
3	PGgtpCY_V	AGGAGGAAGCTTGGCGCTATGGCATCGAAGG
4	PGgtpCY_nco	GCTGGTCGGTTAATGGGTGAAGCTGGG

Table 3

Activity of GTP cyclohydrolase II and riboflavin synthase in *C. guilliermondii* recombinant strains (time of growth: 40-48 h).				
N	Strain	Riboflavin synthase activity nmol mg^{-1} h^{-1}	GTP cyclohydrolase II activity nmol mg^{-1} h^{-1}	Ratio*
1	L2 (wild type)	21.6	2.88	1.00
2	R1-1	n.d.	10.08	3.50
3	R1-2	n.d.	4.20	1.46
4	R1-3	20.4	8.76	3.04
5	R1-4	19.8	7.80	2.70
6	R1-5	21.6	7.80	2.70
7	TC-1	20.4	9.60	3.33
8	TC-2	n.d.	8.40	2.92
9	TC-3	n.d.	7.56	2.63
10	XS-1	22.8	13.38	4.60
11	XS-2	n.d.	12.60	4.37
12	XS-3	n.d.	6.60	2.29

n.d. not determined.

* GTP cyclohydrolase activity of recombinant strain divided by GTP cyclohydrolase activity of strain L2

Table 4

Riboflavin production by recombinant *C. guilliermondii* strains (time of growth: 110 h. incubation temperature: 30 °C).			
N	Strain	Riboflavin production [mg/l]	Relative riboflavin production
1	L2 (wild type)	1.2	1.0
4	R1-3	1.4	1.2
5	R1-4	3.6	3.0
6	R1-5	3.0	2.5
7	TC-1	1.4	1.2
9	TC-3	1.3	1.0
12	XS-3	3.6	3.0
13	XS-4	2.0	1.7
14	XS-5	2.3	1.9

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<120> Overproduction of riboflavin in yeast

<130> Case 20071 EP1

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    50                  55                  60
Lys Cys Met Ala Arg Ala Arg Ile Pro Thr Thr Gln Gly Pro Glu Ile
65                  70                  75                  80
Phe Leu His Leu Tyr Glu Asn Asn Val Asp Asn Lys Glu His Leu Ala
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Ile Val Phe Gly Glu Asp Val Arg Ser Lys Thr Leu Tyr Gln Lys Arg
            100                 105                 110
Pro Asn Glu Thr Gln Gln Asp Arg Met Thr Arg Gly Ala Tyr Val Gly
        115                 120                 125
Arg Leu Phe Pro Gly Arg Thr Glu Ala Asp Tyr Asp Ser Glu Ser Asn
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Thr Thr Cys Ser Gly Glu Pro Ile Leu Ala Arg Ile His Ser Glu Cys
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Tyr Thr Gly Glu Thr Ala Trp Ser Ala Arg Cys Asp Cys Gly Glu Gln
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Phe Asp Glu Ala Gly Arg Leu Met Gly Glu Ala Gly His Gly Cys Ile
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Val Tyr Leu Arg Gln Glu Gly Arg Gly Ile Gly Leu Gly Glu Lys Leu
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Lys Ala Tyr Asn Leu Gln Asp Leu Gly Ala Asp Thr Val Gln Ala Asn
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                245                 250                 255
Ala Ile Leu Leu Asp Leu Gly Leu Asn Glu Ile Lys Leu Leu Thr Asn
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Val Val Glu Arg Val Pro Met Val Pro Leu Ala Trp Arg Ser Glu Asn
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Ala Glu Val Arg Asp Arg Leu Pro Leu Pro Glu Thr Leu Pro Val Val
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Lys Cys Met Ala Arg Ala Arg Ile Pro Thr Thr Gln Gly Pro Glu Ile
65                  70                  75                  80
Phe Leu His Leu Tyr Glu Asn Asn Val Asp Asn Lys Glu His Leu Ala
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            100                 105                 110
Pro Asn Glu Thr Gln Gln Asp Arg Met Thr Arg Gly Ala Tyr Val Gly
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Arg Leu Phe Pro Gly Arg Thr Glu Ala Asp Tyr Asp Ser Glu Ser Asn
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Lys Cys Met Ala Arg Ala Arg Ile Pro Thr Thr Gln Gly Pro Glu Ile
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225                 230                 235                 240
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        35                  40                  45
Ala Glu Val Arg Asp Arg Leu Pro Leu Pro Glu Thr Leu Pro Val Val
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Lys Cys Met Ala Arg Ala Arg Ile Pro Thr Thr Gln Gly Pro Glu Ile
65                  70                  75                  80
Phe Leu His Leu Tyr Glu Asn Asn Val Asp Asn Lys Glu His Leu Ala
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Ile Val Phe Gly Glu Asp Val Arg Ser Lys Thr Leu Tyr Gln Lys Arg
            100                 105                 110
Pro Asn Glu Thr Gln Gln Asp Arg Met Thr Arg Gly Ala Tyr Val Gly
        115                 120                 125
Arg Leu Phe Pro Gly Arg Thr Glu Ala Asp Tyr Asp Ser Glu Ser Asn
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Leu Arg Leu Asn Phe Asp Glu Asn Gly Gln Leu Ile Arg Asp Pro Ser
145                 150                 155                 160
Thr Thr Cys Ser Gly Glu Pro Ile Leu Ala Arg Ile His Ser Glu Cys
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Tyr Thr Gly Glu Thr Ala Trp Ser Ala Arg Cys Asp Cys Gly Glu Gln
            180                 185                 190
Phe Asp Glu Ala Gly Arg Leu Met Gly Glu Ala Gly
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```

transf vs integrated
less than one polypeptide

not yeast
is isolated

Claims

1. A yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promotor functional in such yeast strain. not limited to yeast

2. A yeast strain of claim 1 which belongs to the group of flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation.

3. A yeast strain of claim 2 which is selected from the following group: Schwanniomyces, preferably Schwanniomyces occidentalis, Debaryomyces, preferably Debaryomyces kloeckeri, Torulopsis, preferably Torulopsis candida, or Candida, preferably Candida guilliermondii or Candida famata.

4. The yeast strain of claim 3 which is Candida guilliermondii or Candida famata.

5. A yeast strain as claimed any one of claims 1 to 4 wherein the polypeptide encoding DNA sequence is from yeast, preferably flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation more preferably Candida, e.g. Candida guilliermondii or Candida famata.

6. A yeast strain as claimed in any one of claims 1 to 4, wherein the polypeptide ncoding DNA sequence encodes a

protein with GTP cyclohydrolase II activity and is selected from the following DNA sequences:

a) the DNA sequenc as shown in Fig. 5 or its complementary strand;

b) DNA sequences which hybridize under standard conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and

c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

7. A yeast strain as claimed in any one of claims 1 to 6 wherein the promotor is the TEF S. cerevisiae promotor.

8. A process for the production of riboflavin characterized therein that a yeast strain as claimed in any one of claims 1 to 7 is cultured under suitable culture conditions and the riboflavin produced is isolated from the medium or the yeast strain by methods known to the man skilled in the art.

9. A process for the production of a food or feed composition characterized therein that riboflavin as obtained by the process of claim 8 is mixed with one or more suitable food or feed ingredients.

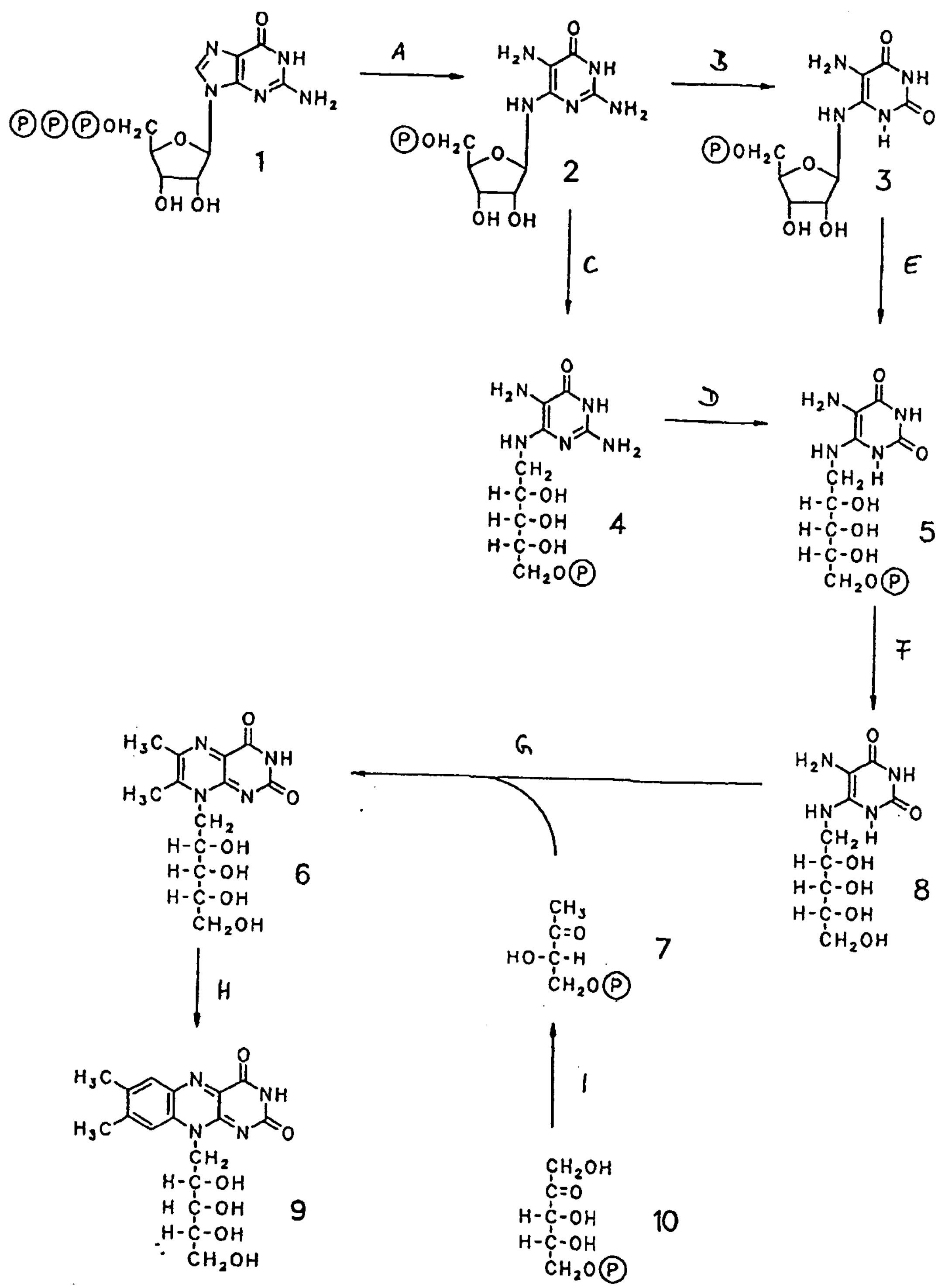

Fig. 1

Fig.2.
Nucleotide sequence of the p19R1 insert.

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         |         |         |         |         |         |
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         |         |         |         |         |         |
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       310       320       330       340       350       360
         |         |         |         |         |         |
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       370       380       390       400       410       420
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CCATGCCTCTCTTATCTCCTACATTGACACCATCCCATATTCCATCGCAAACGCCTCAAA
  METProLeuLeuSerProThrLeuThrProSerHisIleProSerGlnThrProGlnIle
```

```
        610       620       630       640       650       660
          |         |         |         |         |         |
TTCCTCCGGAAGTGCCAGCAGAAGTCAGGGATCGCTTGCCCCTTCCTGAAACGTTGCCTG

  ProProGluValProAlaGluValArgAspArgLeuProLeuProGluThrLeuProVal

        670       680       690       700       710       720
          |         |         |         |         |         |
TGGTGAAATGCATGGCGAGAGCTCGTATACCGACCACTCAGGGGCCGGAGATATTTCTCC

  ValLysCysMETAlaArgAlaArgIleProThrThrGlnGlyProGluIlePheLeuHis

        730       740       750       760       770       780
          |         |         |         |         |         |
ATTTGTACGAGAATAACGTTGACAATAAAGAGCATTTGGCTATTGTTTTGGGGAAGATG

  LeuTyrGluAsnAsnValAspAsnLysGluHisLeuAlaIleValPheGlyGluAspVal

        790       800       810       820       830       840
          |         |         |         |         |         |
TGCGGTCGAAAACGCTCTATCAGAAACGTCCCAATGAGACCCAGCAAGATAGAATGACTC

  ArgSerLysThrLeuTyrGlnLysArgProAsnGluThrGlnGlnAspArgMETThrArg

        850       860       870       880       890       900
          |         |         |         |         |         |
GTGGTGCTTATGTGGGCAGATTGTTTCCTGGAAGAACCGAGGCAGACTATGACAGTGAGT

  GlyAlaTyrValGlyArgLeuPheProGlyArgThrGluAlaAspTyrAspSerGluSer

        910       920       930       940       950       960
          |         |         |         |         |         |
CTAATTTGAGATTGAATTTCGATGAAAATGGCCAACTTATCAGAGATCCGAGTACCACCT

  AsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeuIleArgAspProSerThrThrCys

        970       980       990      1000      1010      1020
          |         |         |         |         |         |
GTAGTGGTGAGCCCATTTTGGCCCGTATTCATTCGGAATGTTATACGGGGGAAACCGCAT

  SerGlyGluProIleLeuAlaArgIleHisSerGluCysTyrThrGlyGluThrAlaTrp

       1030      1040      1050      1060      1070      1080
          |         |         |         |         |         |
GGAGTGCTCGTTGCGATTGTGGAGAACAATTCGATGAAGCTGGTCGGTTAATGGGTGAAG

  SerAlaArgCysAspCysGlyGluGlnPheAspGluAlaGlyArgLeuMETGlyGluAla
```

```
      1090      1100      1110      1120      1130      1140
         |         |         |         |         |         |
CTGGGCACGGGTGTATCGTGTACCTTCGTCAGGAAGGTCGTGGAATTGGACTTGGGGAAA

  GlyHisGlyCysIleValTyrLeuArgGlnGluGlyArgGlyIleGlyLeuGlyGluLys

      1150      1160      1170      1180      1190      1200
         |         |         |         |         |         |
AGTTGAAGGCTTATAATTTGCAAGACTTGGGAGCGGATACCGTCCAGGCCAATTTGATGT

  LeuLysAlaTyrAsnLeuGlnAspLeuGlyAlaAspThrValGlnAlaAsnLeuMETLeu

      1210      1220      1230      1240      1250      1260
         |         |         |         |         |         |
TACGACATCCTGCTGATGCGAGATCTTTTTCGCTCGCTACAGCCATACTCTTGGACTTGG

  ArgHisProAlaAspAlaArgSerPheSerLeuAlaThrAlaIleLeuLeuAspLeuGly

      1270      1280      1290      1300      1310      1320
         |         |         |         |         |         |
GGCTCAACGAGATCAAGTTGTTGACCAACAATCCCGATAAAATTGCTGCAGTTGAGGGAA

  LeuAsnGluIleLysLeuLeuThrAsnAsnProAspLysIleAlaAlaValGluGlyArg

      1330      1340      1350      1360      1370      1380
         |         |         |         |         |         |
GAAACAGAGAGGTCAAGGTAGTGGAACGGGTGCCTATGGTGCCGTTGGCATGGAGAAGTG

  AsnArgGluValLysValValGluArgValProMETValProLeuAlaTrpArgSerGlu

      1390      1400      1410      1420      1430      1440
         |         |         |         |         |         |
AGAATGGAATCAAGTCAAAAGAGATAGAGGGCTACTTGAGTGCTAAGATTGAAAGGATGG

  AsnGlyIleLysSerLysGluIleGluGlyTyrLeuSerAlaLysIleGluArgMETGly

      1450      1460      1470      1480      1490      1500
         |         |         |         |         |         |
GGCACTTGCTTGAAAAGCCACTCAAGATATGATAGAAGAGATGAAGTTAAGGACTTAAGA

  HisLeuLeuGluLysProLeuLysIle------

      1510      1520      1530      1540      1550      1560
         |         |         |         |         |         |
AATAAATGATGAATTAAATGACGCAAATGTCACTACTCGATTAGAGAAATAGCTATAATG

      1570      1580      1590      1600      1610      1620
         |         |         |         |         |         |
AAGAATTTTGCATTTCGCAAAATTTAAGATAAATGCAAAAATTGCAAATTACGAAATATG
```

```
1630      1640      1650      1660      1670      1680
```
CATATGATACAAGACAAGAAAAGACTACTAAAAGTCTCTCGAGAAGAATACTGGGTAACC

```
1690      1700      1710      1720      1730      1740
TTCATCTCTTGATTATGCACTGGGGCTATTCATATGCAGATTCGCACGCCGAGGTGCAGC

1750      1760      1770      1780      1790      1800
GTTTAGGCGCGGCTCAACGGAAGCCAACGGCCGCCACAAATTGTCCGGAAAGTCGCCGAA

1810      1820      1830      1840      1850      1860
ACTGATCCACTGGTACCACAGCCCCATAAGAACCCCCTTTAATATTAAAAACCGTTCTTC

1870      1880      1890      1900      1910      1920
AGCCACTTTTGATCACATTGTTTGCAGCCGCCCGTTGCTGCCATCCAAACACCACGCGTC

1930      1940      1950      1960      1970      1980
CCCCGCACGTTTTACGGTGCCCACTGCATTGGAATTTGCATAAAACAGCCTCACGAAGTG
```
C
```
1990      2000      2010      2020      2030      2040
GATTAATTTTTAGAGCACTCAAGTCATCATGCTGCAATCTCTGCATCATGAAATGACTCC

2050      2060      2070      2080      2090      2100
CGTTGATACAGGGAACTCAGACCGCAAGCGGCGAAGAGTCACAAGAGCGTGTGATGTGTG
```
G
```
2110      2120      2130      2140      2150      2160
TCGACTCTAGAGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTC

2170      2180
GTGACTGGGAAAACCCTGGCG
```

ADM

Sequence of the *RIB1* gene is translated.
Sequence of ARS element is typed in bold letters.

Fig.3
Nucl otide sequence of the pTC2 insert.

```
        10        20        30        40        50        60
         |         |         |         |         |         |
CAATTCGAGCTCGGTACCCGGGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTC

        70        80        90       100       110       120
         |         |         |         |         |         |
CTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC

       130       140       150       160       170       180
         |         |         |         |         |         |
CAAGCACAGCATACTAAATTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTAC

       190       200       210       220       230       240
         |         |         |         |         |         |
TAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCA

       250       260       270       280       290       300
         |         |         |         |         |         |
ATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTGATTTTTTTCTCTTT

       310       320       330       340       350       360
         |         |         |         |         |         |
CGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTTCTCAAGTTTCAGTT

       370       380       390       400       410       420
         |         |         |         |         |         |
TCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTACAAAGAAAGCATAG

       430       440       450       460       470       480
         |         |         |         |         |         |
CAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACCGGCGCCT

       490       500       510       520       530       540
         |         |         |         |         |         |
AGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAG

       550       560       570       580       590       600
         |         |         |         |         |         |
-GAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGCGCCGGCA

                  METAlaSerLysTyrIleValHisProGlnProGluArgArgHis
```

```
       610       620       630       640       650       660
         |         |         |         |         |         |
CGGGTCGGAAACTCACGAATTTACCATGCCTCTCTTATCTCCTACATTGACACCATCCCA

 GlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrProSerHis

       670       680       690       700       710       720
         |         |         |         |         |         |
TATTCCATCGCAAACGCCTCAAATTCCTCCCGAAGTGCCAGCAGAAGTCAGGGATCGCTT

 IleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAspArgLeu

       730       740       750       760       770       780
         |         |         |         |         |         |
GCCCCTTCCTGAAACGTTGCCTGTGGTGAAATGCATGGCGACAGCTCCTATACCGACCAC

 ProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIleProThrThr

       790       800       810       820       830       840
         |         |         |         |         |         |
TCAGGGGCCGGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAGAGCATTT

 GlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGluHisLeu

       850       860       870       880       890       900
         |         |         |         |         |         |
GGCTATTGTTTTTGGGGAAGATGTGCGGTCGAAAACGCTCTATCAGAAACGTCCCAATGA

 AlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgProAsnGlu

       910       920       930       940       950       960
         |         |         |         |         |         |
GACCCAGCAAGATAGAATGACTCGTGGTGCTTATGTGGGCAGATTGTTTCCTGGAAGAAC

 ThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGlyArgThr

       970       980       990      1000      1010      1020
         |         |         |         |         |         |
CGAGGCAGACTATGACAGTGAGTCTAATTTGAGATTGAATTTCGATGAAAATGGCCAACT

 GluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeu

      1030      1040      1050      1060      1070      1080
         |         |         |         |         |         |
TATCAGAGATCCGAGTACCACCTGTAGTGGTGAGCCCATTTTGGCCCGTATTCATTCGGA

 IleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHisSerGlu
```

Fig.4
Nucleotide sequence of the pTCdXS-2 insert.

```
        10        20        30        40        50        60
         |         |         |         |         |         |
CAATTCGAGCTCGGTACCCGGGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTC

        70        80        90       100       110       120
         |         |         |         |         |         |
CTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC

       130       140       150       160       170       180
         |         |         |         |         |         |
CAAGCACAGCATACTAAATTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTAC

       190       200       210       220       230       240
         |         |         |         |         |         |
TAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCA

       250       260       270       280       290       300
         |         |         |         |         |         |
ATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTGATTTTTTTCTCTTT

       310       320       330       340       350       360
         |         |         |         |         |         |
CGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTTCTCAAGTTTCAGTT

       370       380       390       400       410       420
         |         |         |         |         |         |
TCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAG

       430       440       450       460       470       480
         |         |         |         |         |         |
CAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACCGGCGCCT

       490       500       510       520       530       540
         |         |         |         |         |         |
AGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAG

       550       560       570       580       590       600
         |         |         |         |         |         |
GAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGCGCCGGCA

                  METAlaSerLysTyrIleValHisProGlnProGluArgArgHis
```

```
       610       620       630       640       650       660
         |         |         |         |         |         |
CGGGTCGGAAACTCACGAATTTACCATGCCTCTCTTATCTCCTACATTGACACCATCCCA

 GlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrProSerHis

       670       680       690       700       710       720
         |         |         |         |         |         |
TATTCCATCGCAAACGCCTCAAATTCCTCCGGAAGTGCCAGCAGAAGTCAGGGATCGCTT

 IleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAspArgLeu

       730       740       750       760       770       780
         |         |         |         |         |         |
GCCCCTTCCTGAAACGTTGCCTGTGGTGAAATGCATGGCGAGAGCTCGTATACCGACCAC

 ProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIleProThrThr

       790       800       810       820       830       840
         |         |         |         |         |         |
TCAGGGGCCGGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAGAGCATTT

 GlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGluHisLeu

       850       860       870       880       890       900
         |         |         |         |         |         |
GGCTATTGTTTTTGGGGAAGATGTGCGGTCGAAAACGCTCTATCAGAAACGTCCCAATGA

 AlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgProAsnGlu

       910       920       930       940       950       960
         |         |         |         |         |         |
GACCCAGCAAGATAGAATGACTCGTGGTGCTTATGTGGGCAGATTGTTTCCTGGAAGAAC

 ThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGlyArgThr

       970       980       990      1000      1010      1020
         |         |         |         |         |         |
CGAGGCAGACTATGACAGTGAGTCTAATTTGAGATTGAATTTCGATGAAAATGGCCAACT

 GluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeu

      1030      1040      1050      1060      1070      1080
         |         |         |         |         |         |
TATCAGAGATCCGAGTACCACCTGTAGTGGTGAGCCCATTTTGGCCCGTATTCATTCGGA

 IleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHisSerGlu
```

```
      1090      1100      1110      1120      1130      1140
         |         |         |         |         |         |
ATGTTATACGGGGGAAACCGCATGGAGTGCTCGTTGCGATTGTGGAGAACAATTCGATGA

 CysTyrThrGlyGluThrAlaTrpSerAlaArgCysAspCysGlyGluGlnPheAspGlu

      1150      1160      1170      1180      1190      1200
         |         |         |         |         |         |
AGCTGGTCGGTTAATGGGTGAAGCTGGGCACGGGTGTATCGTGTACCTTCGTCAGGAAGG

 AlaGlyArgLeuMETGlyGluAlaGlyHisGlyCysIleValTyrLeuArgGlnGluGly

      1210      1220      1230      1240      1250      1260
         |         |         |         |         |         |
TCGTGGAATTGGACTTGGGGAAAAGTTGAAGGCTTATAATTTGCAAGACTTGGGAGCGGA

 ArgGlyIleGlyLeuGlyGluLysLeuLysAlaTyrAsnLeuGlnAspLeuGlyAlaAsp

      1270      1280      1290      1300      1310      1320
         |         |         |         |         |         |
TACCGTCCAGGCCAATTTGATGTTACGACATCCTGCTGATGCGAGATCTTTTTCGCTCGC

 ThrValGlnAlaAsnLeuMETLeuArgHisProAlaAspAlaArgSerPheSerLeuAla

      1330      1340      1350      1360      1370      1380
         |         |         |         |         |         |
TACAGCCATACTCTTGGACTTGGGGCTCAACGAGATCAAGTTGTTGACCAACAATCCCGA

 ThrAlaIleLeuLeuAspLeuGlyLeuAsnGluIleLysLeuLeuThrAsnAsnProAsp

      1390      1400      1410      1420      1430      1440
         |         |         |         |         |         |
TAAAATTGCTGCAGTTGAGGGAAGAAACAGAGAGGTCAAGGTAGTGGAACGGGTGCCTAT

 LysIleAlaAlaValGluGlyArgAsnArgGluValLysValValGluArgValProMET

      1450      1460      1470      1480      1490      1500
         |         |         |         |         |         |
GGTGCCGTTGGCATGGAGAAGTGAGAATGGAATCAAGTCAAAAGAGATAGAGGGCTACTT

 ValProLeuAlaTrpArgSerGluAsnGlyIleLysSerLysGluIleGluGlyTyrLeu

      1510      1520      1530      1540      1550      1560
         |         |         |         |         |         |
GAGTGCTAAGATTGAAAGGATGGGGCACTTGCTTGAAAAGCCACTCAAGATATGATAGAA

 SerAlaLysIleGluArgMETGlyHisLeuLeuGluLysProLeuLysIle------
```

```
      1570      1580      1590      1600      1610      1620
         |         |         |         |         |         |
GAGATGAAGTTAAGGACTTAAGAAATAAATGATGAATTAAATGACGCAAATGTCACTACT

      1630      1640      1650      1660      1670      1680
         |         |         |         |         |         |
CGATTAGAGAAATAGCTATAATGAAGAATTTTGCATTTCGCAAAATTTAAGATAAATGCA

      1690      1700      1710      1720      1730      1740
         |         |         |         |         |         |
AAAATTGCAAATTACGAAATATGCATATGATACAAGACAAGAAAAGACTACTAAAAGTCTCT
```

Sequence of the *TEF* promoter is underlined.
Sequence of the *RIB1* gene is translated.
Sequence of ARS element is typed in bold letters.

Fig.5

Nucleotide sequence of PCR product obtained with primers ShBle_V and PGgtpCY_nco using boiled cells of *P.guilliermondii* XS-3 recombinant strain.

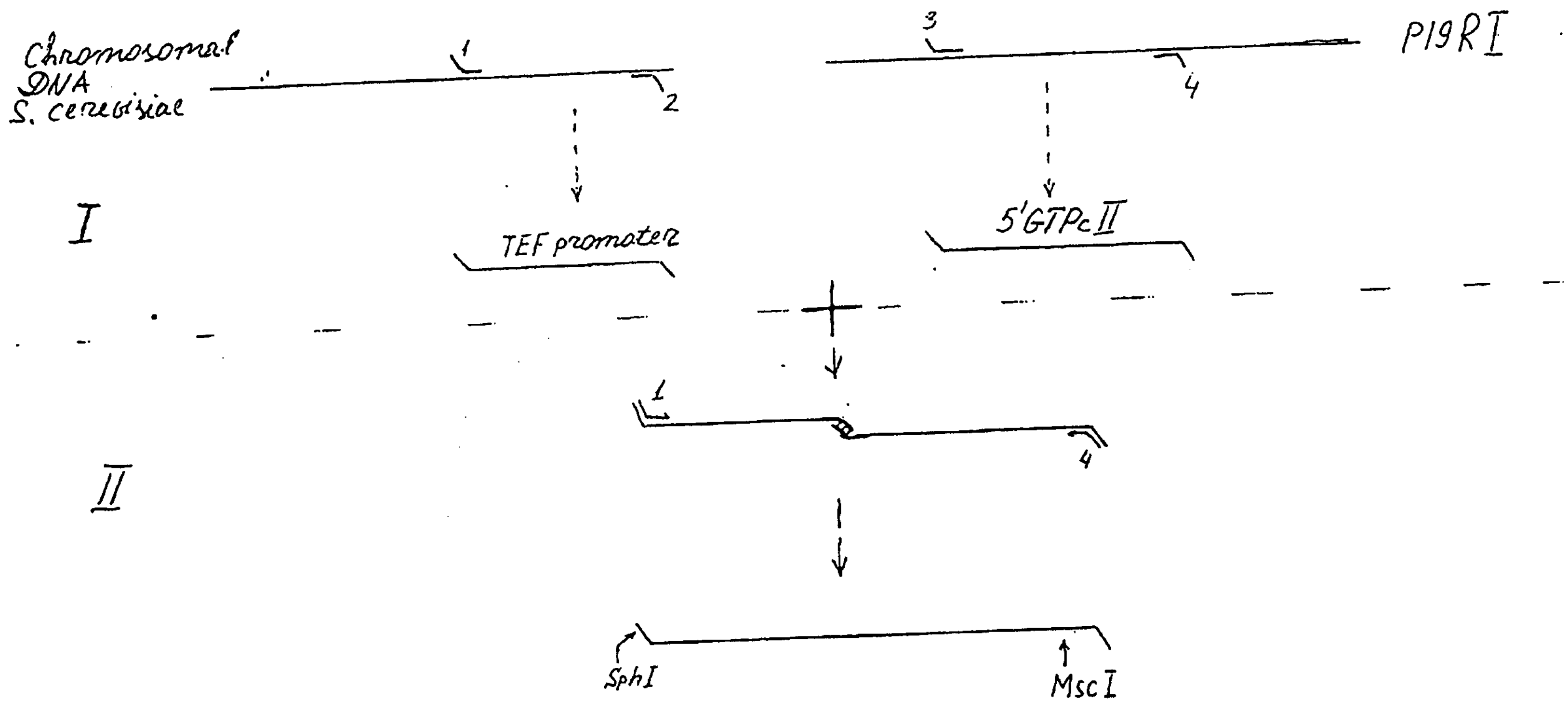
```
        10        20        30        40        50        60
         |         |         |         |         |         |
GGGCATGCAATTCGAGCTCGGTACCCGGGGATCCCCCACACACCATAGCTTCAAAATGTT

        70        80        90       100       110       120
         |         |         |         |         |         |
TCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCA

       130       140       150       160       170       180
         |         |         |         |         |         |
AAACACCCAAGCACAGCATACTAAATTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTA

       190       200       210       220       230       240
         |         |         |         |         |         |
CCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAA

       250       260       270       280       290       300
         |         |         |         |         |         |
AAAGGCAATAAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTGATTTTT

       310       320       330       340       350       360
         |         |         |         |         |         |
TCTCTTTCGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTTCTCAAGT

       370       380       390       400       410       420
         |         |         |         |         |         |
TTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAA

       430       440       450       460       470       480
         |         |         |         |         |         |
AGCATAGCAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACC

       490       500       510       520       530       540
         |         |         |         |         |         |
GGCGCCTAGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTC

       550       560       570       580       590       600
         |         |         |         |         |         |
ACTATAGGAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGC

                                 METAlaSerLysTyrIleValHisProGlnProGluArg
```

```
      610       620       630       640       650       660
        |         |         |         |         |         |
GCCGGCACGGGTCGGAAACTCACGAATTTACCATGCCTCTCTTATCTCCTACATTGACAC

  ArgHisGlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrPro

      670       680       690       700       710       720
        |         |         |         |         |         |
CATCCCATATTCCATCGCAAACGCCTCAAATTCCTCCGGAAGTGCCAGCAGAAGTCAGGG

  SerHisIleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAsp

      730       740       750       760       770       780
        |         |         |         |         |         |
ATCGCTTGCCCCTTCCTGAAACGTTGCCTGTGGTGAAATGCATGGCGAGAGCTCGTATAC

  ArgLeuProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIlePro

      790       800       810       820       830       840
        |         |         |         |         |         |
CGACCACTCAGGGGCCGGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAG

  ThrThrGlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGlu

      850       860       870       880       890       900
        |         |         |         |         |         |
AGCATTTGGCTATTGTTTTTGGGGAAGATGTGCGGTCGAAAACGCTCTATCAGAAACGTC

  HisLeuAlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgPro

      910       920       930       940       950       960
        |         |         |         |         |         |
CCAATGAGACCCAGCAAGATAGAATGACTCGTGGTGCTTATGTGGGCAGATTGTTTCCTG

  AsnGluThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGly

      970       980       990      1000      1010      1020
        |         |         |         |         |         |
GAAGAACCGAGGCAGACTATGACAGTGAGTCTAATTTGAGATTGAATTTCGATGAAAATG

  ArgThrGluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGly

     1030      1040      1050      1060      1070      1080
        |         |         |         |         |         |
GCCAACTTATCAGAGATCCGAGTACCACCTGTAGTGGTGAGCCCATTTTGGCCCGTATTC

  GlnLeuIleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHis

     1090      1100      1110      1120      1130      1140
        |         |         |         |         |         |
ATTCGGAATGTTATACGGGGGAAACCGCATGGAGTGCTCGTTGCGATTGTGGAGAACAAT

  SerGluCysTyrThrGlyGluThrAlaTrpSerAlaArgCysAspCysGlyGluGlnPhe
```

```
     1150      1160      1170
        |         |         |
TCGATGAAGCTGGTCGGTTAATGGGTGAAGCTGGG

  AspGluAlaGlyArgLeuMETGlyGluAlaGly
```

Sequence of the *TEF* promoter is underlined.
Partial sequence of the *RIB1* gene is translated.

Scheme
of the 2-steps PCR amplification



1. ShBle_V
2. TEF1_H
3. PGgtpCY_V
4. PGgtpCY_nco